Ultrastructural Changes Resulting from Keratin-9 Gene Mutations in Two Families with Epidermolytic Palmoplantar Keratoderma

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Palmoplantar keratoderma of Voerner type (or epidermolytic palmoplantar keratoderma) is an autosomal dominant inherited disorder of keratinization with histologic features of epidermolytic hyperkeratosis. We studied members of two large unrelated kindreds with epidermolytic palmoplantar keratoderma, and biopsy specimens of lesional palmar skin from both families confirmed the histologic changes of epidermolytic hyperkeratosis. Whorls of abnormally aggregated keratin filaments were seen ultrastructurally to be associated with signs of cellular disintegration in spinous and granular cells. Direct sequencing of genomic DNA samples obtained from several members of each family established the substitution of a highly conserved arginine by tryptophan (R162W) in the 1A region of the alpha-helical rod domain of keratin 9. This arginine residue in a highly conserved region of keratins 1 and 10 is affected by disruptive missense point mutations in many patients with bullous ichthyosiform erythroderma. An equivalent position in the sole and palm restricted keratin 9 appears to be the mutation hot spot in epidermolytic palmoplantar keratoderma. To date, R162W is the most prevalent genetic defect reported in this genodermatosis. *J Invest Dermatol 104:425–429, 1995*

The palmoplantar keratodermas (PPK) are a heterogeneous group of conditions most frequently inherited in an autosomal dominant fashion, with a few well-documented autosomal recessive disorders, or acquired in association with certain metabolic disorders and malignancies. The epidermolytic type of palmoplantar keratoderma (EPPK, or Voerner's syndrome), is characterized clinically by diffuse thickening of the skin over the palms and soles, with a well-demarcated erythematous border [1,2]. Blistering or peeling is uncommon, although histopathology shows epidermolytic hyperkeratosis (EHK), the key pathologic feature of congenital bullous ichthyosiform erythroderma and other epidermolytic ichthyoses (such as ichthyosis bullosa of Siemens), which have been shown to result from keratin gene mutations of K1/K10 and K2, respectively [3–10]. PPK is a prominent feature of both K1 and K10 gene mutations in some patients [11], so keratin genes appear likely candidate genes in hereditary EPPK. The candidacy of K9, a type I keratin, was strongly supported by its specific expression in palmoplantar epidermis [12] and was confirmed by recent molecular genetic studies. A gene for EPPK was initially mapped to the region of the acidic (type I) keratin gene cluster at 17q12–21 in a large kindred [13]. Recently, different point mutations of the K9 gene have been identified in unrelated families with EPPK [14–16].‡

The hereditary nonepidermolytic forms of PPK (NEPPK) show a variety of patterns and associated features [17]. These include diffuse NEPPK, focal NEPPK, and punctate NEPPK. Diffuse NEPPK (or tylosis) has been reported in association with a high risk of developing carcinoma of the esophagus (Howel-Evans syndrome) [18,19], and punctate NEPPK has been associated with a high risk of other malignancies [20], but the majority of cases of hereditary NEPPK are not associated with carcinoma. Recent reappraisal of Thost's family showing that they had EPPK, not NEPPK [21], has emphasized the importance of histopathologic investigation in the differential diagnosis of NEPPK and EPPK.

We have had the opportunity to study two large, unrelated kindreds with biopsy-proved EPPK, and we report here cutaneous ultrastructural observations and genetic analysis showing a missense mutation of the codon encoding a highly conserved arginine in the K9 gene.

MATERIALS AND METHODS

Patients

Family 1: The proband was a 48-year-old Greek man (IV11), who developed confluent waxy thickening with fissuring of the skin of the palms

Manuscript received July 18, 1994; revised December 9, 1994; accepted for publication December 12, 1994.

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Abbreviations: EPPK, epidermolytic palmoplantar keratoderma; NEPPK, nonepidermolytic palmoplantar keratoderma; PPK, palmoplantar keratoderma.

‡ Bowden PE, Watts CE, Marks R: Mutation of human keratin 9 (HK9) gene in epidermolytic tylosis (abstr). *J Invest Dermatol* 102:576, 1994; Wojczik S, Rothnagel JA, Hohl D, Roop DR: Mutation of a critical arginine residue within the 1A segment of K9 in epidermolytic palmoplantar keratoderma (abstr). *J Invest Dermatol* 102:541, 1994.

0022-202X/95/$09.50 • SSDI0022-202X(94)00371-D •

and soles at the age of 3 months. He had a history of blistering in childhood, but not in later life. The condition was inherited from his father through the maternal line, and the patient had two affected sons. Over 40 members of this Greek family are alive and exhibit autosomal dominant disease inheritance **(Fig 1*a*)**. Clinical examination of eight affected members of the family revealed gross palmoplantar hyperkeratosis with a clear erythematous demarcation and without transgradiens spread. The skin was normal at other sites, including extensor and flexor surfaces, as were the oral mucosa, teeth, and nails. There was no significant intrafamilial variation in the clinical pattern of disease.

Family 2: This English family appeared clinically similar to family 1, with minimal intrafamilial variation. The proband, a 52-year-old English woman (IV2) with no Greek relatives, developed PPK shortly after birth. She gave no history of blistering, although blisters had been noted in both of her affected children. The condition was inherited from her mother through the paternal line **(Fig 1*b*)**. Examination of six affected family members revealed diffuse, uniform keratoderma of the palms and soles without any associated systemic disorders.

Samples After receiving informed consent, we obtained blood samples from affected and unaffected family members and 4-mm punch biopsy specimens from the hypothenar region of lesional palmar skin from one affected member in each family. Tissue samples were processed for light microscopy as well as transmission electron microscopy. Five-micrometer sections of formalin-fixed, paraffin-embedded material or of unfixed snap-frozen tissue were cut and processed for hematoxylin and eosin staining.

Extraction of Genomic DNA Genomic DNA was extracted from whole blood using the high-salt method [22]. DNA was extracted from six members of family 1 (of whom four were affected and two unaffected) and from five members of family 2 (four affected and one unaffected). Genomic DNA was also extracted from 30 unrelated individuals from the normal population and used as controls.

Polymerase Chain Reaction (PCR) A 650-base pair genomic DNA fragment of the K9 gene [12] was amplified by primers 5′AGCCGGTAGCACTCCTATCA3′ (designated K9P1, sense strand) and 5′GTTCTTCTGGATAGCAGCAGG3′ (designated K9P2R, antisense strand). The primer K9P2R was biotinylated at the 5′ end. Approximately 500 ng of genomic DNA was used per 100 μg reaction product containing 1.5 mM $MgCl_2$, 0.1 mM each dNTP, 10% dimethylsulfoxide, 0.5 μg/ml for each primer, and 2.5 units of Taq polymerase (AmpliTaq). After an initial incubation at 94°C for 5 min, PCR was performed for 30 cycles consisting of 94°C for 30 seconds, 55°C for 1 min, and 72°C for 1 min on a DNA Thermal Cycler (Hybaid Ltd.). An additional step of 72°C for 5 min was performed at the end of the program. The PCR product was visualized on 1% agarose gels with ethidium bromide staining.

Single-Stranded Sequencing Another primer designated K9P3 (5′GGAGGAGGTGATGGTGGTAT3′) was used as an internal sequencing primer. The double-stranded DNA product of PCR was purified by extraction with phenol:chloroform (1:1) and then with chloroform. The DNA was precipitated using a 0.5 volume of 7.5 M NH_4OAc and 2 volumes of ethanol at −20°C for more than 4 h. The double-stranded DNA from each reaction was resuspended in 200 μl of diethyl polycarbonate–treated water. This is sufficient for two sequencing reactions using single-stranded sequencing with Streptavidin Dynal M-280 beads (Dynal, UK). Sequencing reactions were conducted using the USB Sequenase II kit. The sequencing gels were exposed to x-ray film for 3–5 d before developing.

Transmission Electron Microscopy The tissue was processed for electron microscopy in a standard fashion [23]. Briefly, primary fixation was for 72 h at +4°C in half-strength Karnovsky fixative. Specimens were post-fixed in 1.3% osmium tetroxide in distilled water for 2 h at +4°C. After dehydration in a graded ethanol series, specimens were embedded in epoxy resin (Taab 812) via propylene oxide. Semithin sections (0.5 μm) and ultrathin sections (60–90 nm) were cut on a Reichert OMU-4 ultramicrotome. Semithin sections were stained with Richardson stain for light microscopy. Ultrathin sections were collected on pioloform-coated copper grids and double stained with 5% uranyl acetate in ethanol and Reynolds lead citrate. Sections were viewed using a JEOL 100 CX transmission electron microscope.

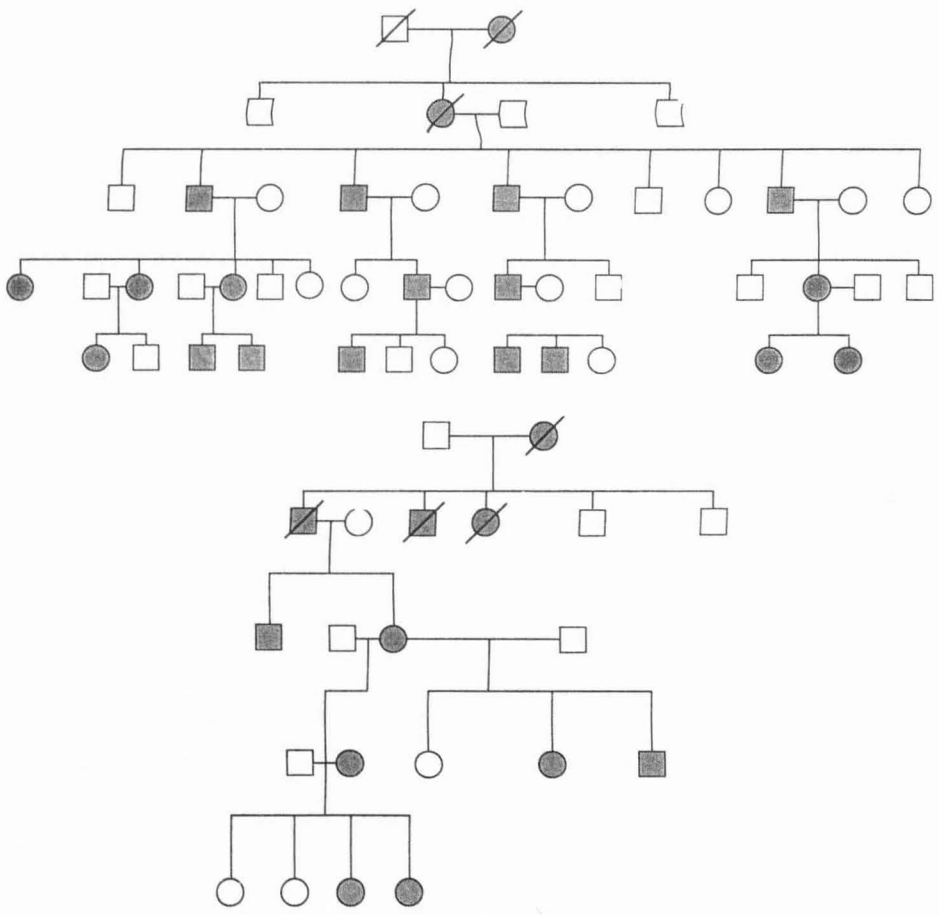

Figure 1. Pedigrees of family 1 (*top*) and family 2 (*bottom*) with EPPK. *Shaded circles*, affected female; *open circles*, normal female; *shaded squares*, affected male; *open squares*, normal male.

RESULTS

Light Microscopy Shows Unusual Conical Spikes of Palmoplantar Epidermis In contrast to normal palm skin **(Fig 2*A*)**, lesional palm skin from both families revealed changes characteristic of EHK **(Fig 2*B*)**. There was marked hyperkeratosis, acanthosis, and papillomatosis. Keratinocytes throughout the spinous and granular layers contained darkly stained fibrillar material, densely aggregated in places. Numerous cells appeared vacuolated and disrupted. The intradermal and intraepidermal portions of the eccrine ducts appeared uninvolved. Lesional epidermis of patient IV11 (pedigree I) showed a distinctive conical or spike-like arrangement of the upper spinous and granular layers extending into the hyperkeratotic stratum corneum **(Fig 2*C*)**.

Suprabasal Tonofilaments Are Highly Abnormal in EPPK Basal keratinocytes of lesional epidermis were ultrastructurally normal. A major change in the spinous and granular epidermal layers was the formation of irregularly shaped whorls of fibrillar material composed of abnormally aggregated keratin filaments. These aggregates became increasingly prominent from the lowermost suprabasal layer to the upper spinous and granular layers **(Fig**

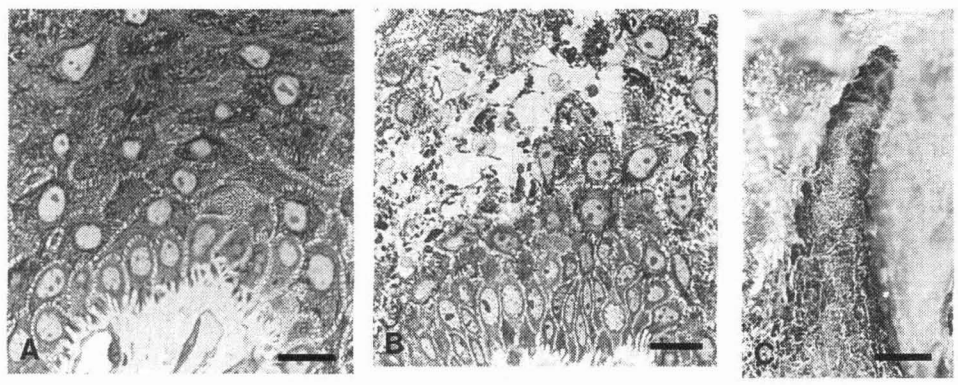

Figure 2. Suprabasal keratinocytes are disrupted in EPPK. Light microscopic comparison between normal (*A*) and lesional (*B,C*) palmar skin shows marked tonofilament changes and keratinocyte vacuolization in epidermis affected by EPPK. *A*) Basal cells in normal palmar epidermis above dermal papilla show typical digit-like basal protrusions. Note dense fibrillar material within spinous cells. Richardson stain. *Bar*, 10 μm. *B*) Acanthotic palmar epidermis. The basal layer appears normal. In the overlying spinous layers, areas of vacuolization are seen next to varying amounts of darkly staining aggregated material. Richardson stain. *Bar*, 10 μm. *C*) Conical or spike-like extension of granular and spinous cell layers into hyperkeratotic stratum corneum. Cryostat section; hematoxylin and eosin stain. *Bar*, 20 μm.

3*A*). The cells that appeared vacuolated and edematous by light microscopy were characterized by large areas of filament-free cytoplasm containing prominent polyribosomes. The abnormal aggregates or whorls also were present and tended not to associate with desmosomes, which were relatively sparse **(Fig 3*B,C*)**. Many cells appeared to be disintegrating, with frank disruption of plasma membranes. Keratohyalin granules were not overtly abundant. In places, keratohyalin was associated with keratin filaments as in normal palm and sole skin, yet more often round or oval profiles of homogeneously electron-dense keratohyalin were surrounded by polyribosomes. In some places, the abnormal keratin aggregates persisted into lower parts of the stratum corneum. Apart from these changes, the stratum corneum appeared intact morphologically and revealed no disturbance of cohesion between corneocytes.

K9 Sequencing Shows Missense Mutations A 650-base pair fragment of genomic DNA including the coding sequence of the helical 1A domain of the rod region of K9 was amplified by PCR. No differences were observed between the sequence obtained and the published sequence of K9 in 30 normal controls [23]. All four affected members of family 1 were found to be heterozygous for a T_{486} C transition in codon 162 of K9, which thus encoded a tryptophan (TGG) instead of the normal arginine (CGG) residue **(Fig 4)**. An identical mutation was detected in the four affected members of family 2, but not in three unaffected members from both families. The base change was confirmed by sequencing the reverse strand using the K9P2R primer. This mutation does not create or destroy any of the recognition sites for restriction enzymes. As a result, rapid screening for this mutation in the general population was not possible using restriction fragment length polymorphisms. As stated above, 30 controls from the normal population were sequenced with the same primers, and no mutation was detected in any of these samples.

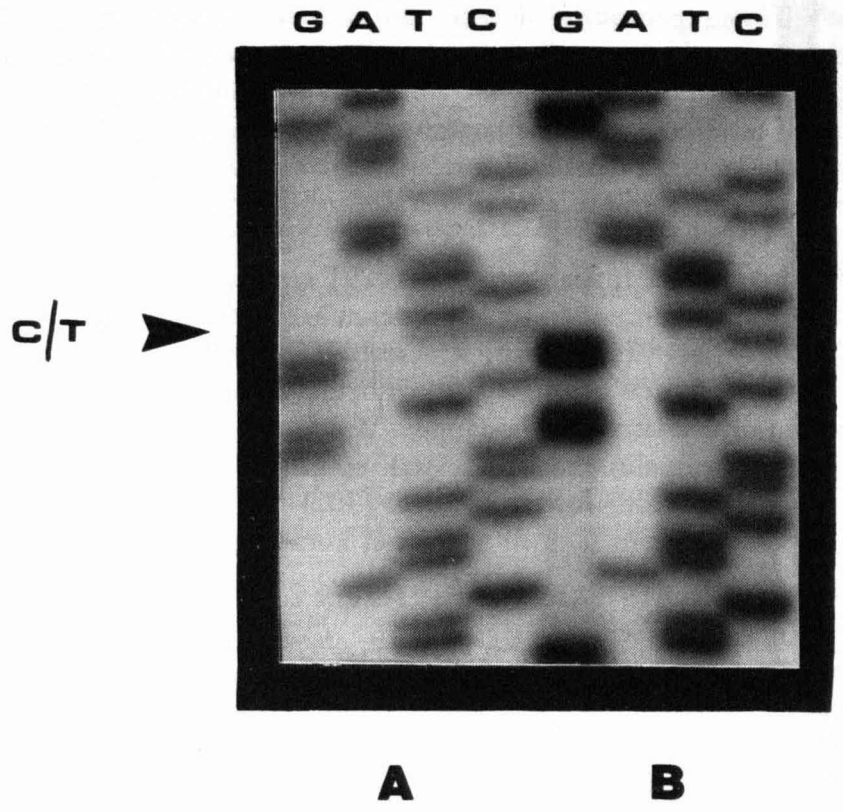

Figure 4. K9 mutations are found in sequencing gels of genomic DNA. Sequencing gel from an affected family member (pedigree 1V11) and an adjacent unaffected member. *Arrow* indicates a C-T transition, which codes for tryptophan (TCG at codon 162). The same region of an unaffected member, as in the normal population, codes for arginine (CGG) at codon 162 in the 1A region of K9.

Although the families were of different racial origin, linkage analysis was performed (David Kelsall) in pedigree 1 using DNA from 12 affected and four unaffected family members, using a PCR-based technique capable of resolving alleles that differed in size by 1 base pair [24].§ The disease gene clearly segregated with a number of polymorphic markers for the acidic keratin cluster on chromosome 17q, including a CA repeat unit from within the K9 gene. Analysis of four affected and two unaffected members of pedigree 2 showed segregation of a different-size K9 allele with the disease, making it highly unlikely that the two families had inherited the same ancestral mutation (not shown).

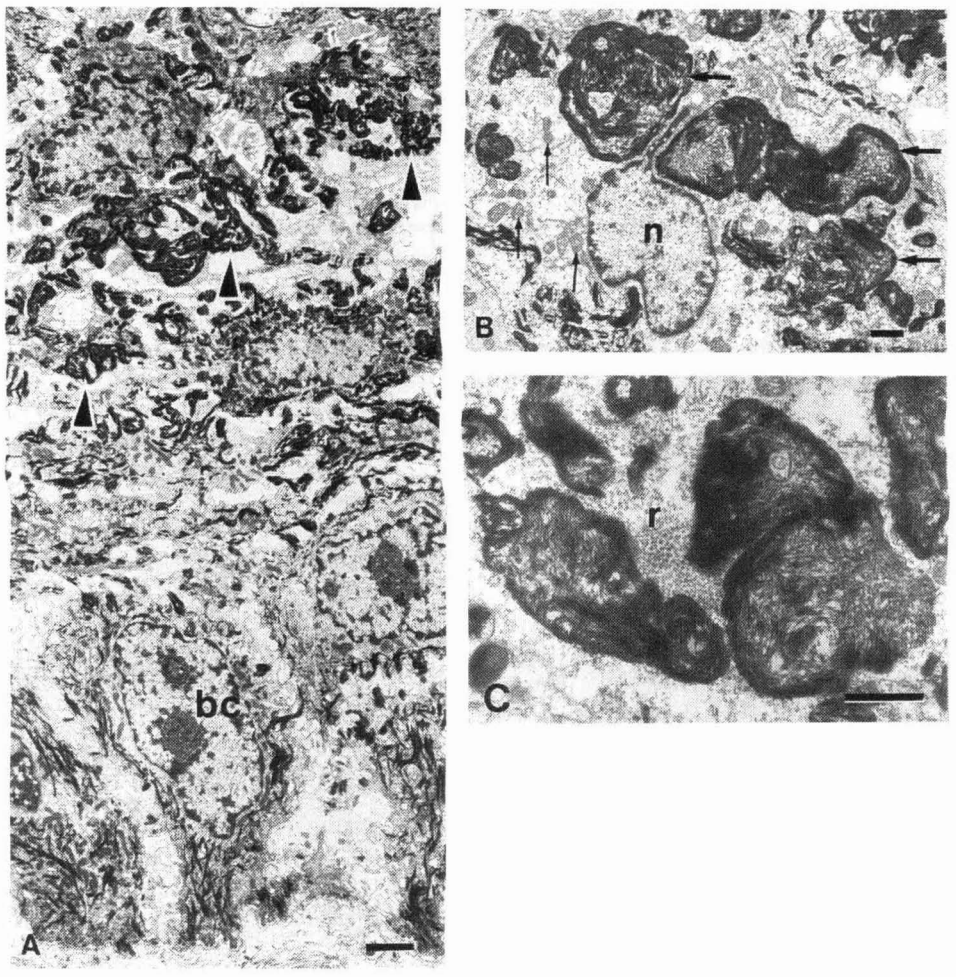

Figure 3. Keratin filaments are abnormally aggregated in suprabasal keratinocytes. Transmission electron microscopy of lesional palmar EPPK epidermis. *A*) Overview illustrating the transition from normal-appearing basal cells (bc) to keratinocytes of the lower and middle spinous layers, which contain increasing amounts of clumped fibrillar material (*arrowheads*). *Bar*, 2 μm. *B*) Individual keratinocyte of the upper spinous layer showing abnormal aggregates and whorls of filamentous material (*large arrows*). Despite marked intracellular edema and almost complete disintegration of the cytoskeleton, mitochondria (*small arrows*) appear well preserved. Part of the nucleus (n) appears compressed between the filament aggregates. *Bar*, 1 μm. *C*) Higher magnification of keratin-filament aggregates and adjacent polyribosomes (r). *Bar*, 1 μm.

DISCUSSION

Point mutations in the genes encoding epidermal keratins have been identified as underlying a number of hereditary skin diseases [3–11]. Voerner type EPPK is the first PPK with an identifiable keratin-gene mutation [14–16]. To date, there have been few reports on the ultrastructural morphology of EHK as seen in EPPK [25–29]. Using transmission electron microscopy, we found that in lesional skin the basal keratinocytes appeared normal, whereas suprabasal cells showed whorls of abnormally aggregated keratin-filament bundles. Spinous and granular cells almost completely lacked normal keratin filaments and in places revealed signs of cellular disintegration, with frank disruption of plasma membranes.

The Extent of Cellular Disruption is Greater in EPPK Than in Other Forms of EHK Both cases studied revealed signs of disruption of the keratin-filament network to a degree not usually seen in lesional skin from congenital bullous ichthyosiform erythroderma or ichthyosis bullosa of Siemens [1,3,7]. The pronounced ultrastructural changes depicted here may be explained in part by the distinct and characteristic arrangement of the keratin-filament network of palmar epidermis, not usually seen in skin from other

§ Kelsell DP, Stevens HP, Ratnavel R, Bryant SP, Bishop DT, Leigh IM, Spurr NK (manuscript in preparation).

sites.¶ These profound defects only very occasionally produce blister formation or clinical shedding of hyperkeratotic areas [26,29]. In addition, normal palmoplantar epidermis expresses multiple other keratin pairs suprabasally apart from K9.** Whether these other keratins may compensate for the faulty K9 or whether more than one keratin is involved in forming the keratin clumps is unknown.

Whereas some investigators [27–29] reported clumping and a shell-like arrangement of tonofilaments around keratinocyte nuclei in EPPK, others [25,26] also stressed an increase in granular cells and the appearance of huge and distorted keratohyalin granules as being major ultrastructural changes. We found no major abnormalities of keratohyalin granules. Such round or oval granules have been noted in other forms of EHK [30,31], and their presence is likely to result from lack of a normal keratin-filament matrix.

R162W is the Most Common Defect to Date in EPPK The point mutation of wild-type codon 162 (CGG) produces the mutant codon TGG, leading to substitution of arginine by tryptophan (R162W). This substitution emerges as the most common genetic defect reported to date in EPPK. This is a nonconservative change consistent with the methyl CpG deamination [32] mechanism of mutation, which accounts for some 90% of human point mutations. It appears that few changes are tolerable in this region, as other reported mutations include a different substitution of the same arginine residue to glutamine (CAG, R162Q) and point mutations of the adjacent codon 160 (AAT, asparagine), producing substitutions to lysine (AAA) and tyrosine (TAT) (N160K, N160T) [14–16]. None of these nucleotide variants were observed in the 30 genomic samples from our unaffected (related and unrelated) controls.

The observed clustering of mutations in EPPK is similar to that seen in the genes encoding basal K5/K14 and suprabasal K1/K10 in epidermolysis bullosa simplex and generalized bullous congenital ichthyosiform erythroderma, respectively [3–11]. The evolutionarily highly conserved arginine of the 1A region of the alpha-helix is thus the most common site of mutations in all disorders of keratinization, reflecting the probable importance of the amino terminus of the central rod in maintenance of the normal helical-helical interactions in keratin proteins [33–35]. Among all the type 1 keratins, K9 shows least conservation in the 1A domain, probably a sign of specialization. There are important questions regarding the pairing of K9 in the epidermis, such as whether it really forms filaments with K1 or whether it can only do so if K10 is also present.

The hereditary breast-ovarian cancer gene locus (BRCA-1) also has been found on chromosome 17q. A large French family with EPPK includes eight women with carcinoma of the breast, ovary, or both [25]. Although a mutation in the K9 gene has been demonstrated in four affected individuals, the cancer risk is thought to be related to changes in a closely linked tumor suppressor gene—possibly the BRCA-1 locus. The association with PPK then results from two separate genetic events of closely linked genes that are cosegregated within the family [15]. Similar mechanisms may present in other PPKs.

Little Evidence so Far for Keratin Mutations in NEPPK There is only a small amount of evidence as yet to show that diffuse NEPPK results from point mutations in keratin genes. A Ukrainian family has been reported with NEPPK showing linkage to the keratin cluster on chromosome 17 [36]. Linkage analysis in a large family with NEPPK in association with esophageal cancer (the original Howel Evans family) clearly excluded linkage to the keratin gene clusters but did segregate to a locus on chromosome 17q23 [37]. In these families, hyperplasia of the palms and soles in NEPPK could result from abnormalities in growth factors or their receptors, or changes in the regulation of keratin genes as well as of other structural proteins. Therefore, recent reports show clearly that PPKs are biologically as well as clinically heterogeneous. EPPK, however, appears to result predominantly from mutations in the highly conserved 1A region of K9, particularly in the arginine residue, which is the major hot spot for mutation in congenital bullous ichthyosiform erythroderma [33–35]. The observed clustering of mutations facilitates the development of oligonucleotide probes to determine the presence of disease-associated mutations in microscopic quantities of genomic material, of potential value in prenatal diagnosis and gene therapy.

¶ Swensson O, Eady RAJ: Ultrastructural analysis of normal human palm and sole skin: an important prerequisite to studying palmoplantar keratoderma and related disorders (abstr). *J Invest Dermatol* 103:260, 1994.

** Swensson O, McMillan JR, Churchill L, Egelrud T, Leigh IM, Eady RAJ: Immunolabelling studies for keratins in normal human palm and sole skin: another prerequisite for studying palmoplantar keratoderma (abstr). *J Invest Dermatol* 103:865, 1994.

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